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Comment

Seeking Common Ground in Nuclear Complexity

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On a molecular scale, the nucleus is a big place. And, due to the enormous mass of DNA, RNA, and protein concentrated there, the nucleus is far denser than the cytoplasm. So the question of how various nuclear factors move about and efficiently find their sites of action has long been a subject of interest and debate. This biological problem is complicated by the fact that most such factors function in large macro-molecular assemblies, with as many as 100 components that must interact at the right time and place. Relevant to this, many factors, including splicing factors, are both dispersed through the nucleoplasm and concentrated in certain nuclear compartments (Fig. 1). Two recent studies, one in *The Journal of Cell Biology* (Kruhlak et al., 2000) and the other in *Nature* (Phair and Misteli, 2000) provide new insights into this fundamental question. Both studies investigate the nuclear dynamics of green fluorescent protein (GFP)[1]–labeled proteins, particularly splicing factor ASF/SF2, in living cells using FRAP (fluorescence recovery after photobleaching) and FLIP (fluorescence loss in photobleaching) to ask the question: how fast do splicing factors move within the nucleus? The answer to this question is significant for understanding the mechanisms by which nuclear factors reach their sites of function. Do the kinetics of factor movement suggest that they are freely diffusing or constrained by structure, perhaps actively recruited from one place to the next? Is ASF/SF2 in the dispersed nucleoplasmic pool, thought to be the most active fraction, more mobile than in the highly concentrated regions frequently considered to be factor storage sites?

Do Nuclear Factors Move Quickly or Slowly?

Both Kruhlak et al. (2000) and Phair and Misteli (2000) find that, irrespective of where it is in the nucleus, GFP-ASF/SF2 shows a high degree of mobility, with ~10% in an immobile fraction. With a diffusion coefficient of 0.24 $\mu m^2 s^{-1}$, it would thus take about a minute to move halfway across the nucleus and five minutes to equilibrate throughout. Phair and Misteli (2000) find slightly higher mobilities for GFP-fibrillarin and the tagged chromatin protein HMG-17. An important point in both studies is that GFP alone moves ~100-fold faster than the GFP-tagged nuclear proteins, so fast that the photobleached zone could not be photographed before significant recovery.

A couple of technical considerations of these experiments merit mention. The presence of higher protein levels, due to expression of exogenous protein, could raise the apparent mobility of nuclear proteins if endogenous binding sites become saturated as a consequence. As Kruhlak et al. (2000) indicate, nucleoplasmic levels of GFP-ASF often appear higher than in immunofluorescence for the endogenous splicing factors. The presence of the GFP tag might also cause an increase in mobility, if it weakens or disrupts normal interactions. Importantly, GFP-histone H2B shows very little movement in nuclei (Phair and Misteli, 2000, and references therein), indicating that the GFP moiety does not confer mobility to just any protein.

Irrespective of any caveats, the two studies apply this novel approach to obtain convincing and concurring estimates for the mobility rates of the proteins studied. So given these results, does GFP-ASF/SF2 move quickly or slowly? Is it diffusing or not? As is often the case, the answer may lie in the eye of the beholder. Both studies make the important point that there is substantial mobility of proteins studied, irrespective of the nuclear compartment, which has further implications discussed below. But despite very similar data, there are notable differences in interpretation and emphasis. Though not necessarily incompatible (see below), they evoke long-standing questions as to the fundamental nature of the nucleoplasm. Kruhlak et al. (2000) state that movement is much slower than expected for free diffusion, based on the kinetics of GFP movement alone. The comparison to GFP is key, as it provides a baseline for how fast a soluble protein can move through nuclear viscosity. But the comparison is an imperfect one, because proteins differ in their size and charge. Both studies state that the twofold greater mass of ASF/SF2 itself would not account for the 100-fold reduction in mobility. Neither group finds major differences in rates of movement in transcriptionally inhibited cells, suggesting that binding to nascent transcripts is not the major factor in reducing mobility of ASF/SF2. So what accounts for the reduction in ASF/SF2 movement? Phair and Misteli (2000) conclude that the rates are consistent with a diffusing protein that is associated with "other components of "nucleoplasmic space," suggesting that "putative structural elements" are "insufficient to retard the movement of studied proteins." On the other hand, Kruhlak et al. (2000)

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[1]*Abbreviations used in this paper:* FLIP, fluorescence loss in photobleaching; FRAP, fluorescence recovery after photobleaching; GFP, green fluorescent protein.

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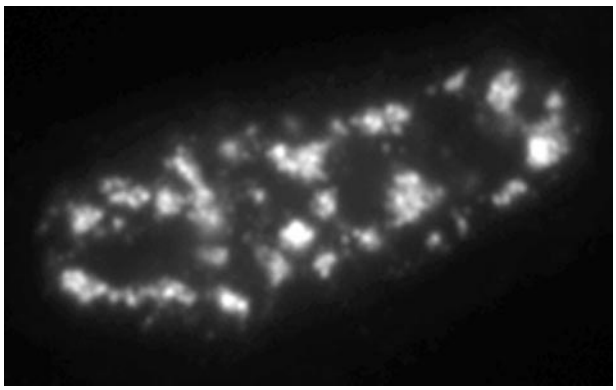

Figure 1. Most splicing factors distribute at lower levels through the nucleoplasm and concentrate in ~20–40 distinct domains, forming a characteristic speckled pattern. Shown is a human fibroblast stained with anti-SC-35, a spliceosome assembly factor (Yeakley et al., 1999, and references therein).

suggest that reduced movement of ASF/SF2 supports its "frequent but transient association with relatively immobile binding sites." In their discussion they go further to suggest that this is evidence of an association with a non-chromatin, insoluble nuclear matrix or karyoskeleton.

Is ASF/SF2 Associated with a Nuclear Matrix?

Given the often polarized views on the existence of a nuclear matrix, the interpretation by Kruhlak et al. (2000) will likely be a controversial one. Rather than polarize this subject further by emphasizing potential differences in interpretation, we offer a perspective that might identify a common ground. There is a great deal of evidence supporting the existence of insoluble, non-chromatin structural elements in the nucleus, and some components of this broadly defined nuclear matrix have indeed been identified (for example, see Blencowe et al., 1994; Cordes et al., 1993). This is not to say that the existence of a system of long cytoskeletal-like filaments has been proven in vivo and biochemically characterized. To our understanding, this definition of nuclear matrix as long homopolymer filaments is more narrow and demanding than that conveyed in most papers by leading investigators in this area. Rather, morphological evidence of short core filaments, resolvable by electron microscopy, has been presented in the context of a subcomponent of much more heterogeneous fibrillogranular material of the nuclear matrix (for reviews, see Fey et al., 1991; Stein and Berezney, 1996). Thus, the nuclear matrix can be conceived of as a dense meshwork of large macromolecular complexes that may include not only diverse proteins but RNAs as well, such as the non-coding XIST RNA involved in X inactivation.

How does this relate to the interpretations and implications of the recent papers from Hendzel's and Misteli's labs? At some point the large macromolecular complexes with which ASF/SF2 interacts may associate with each other or other components of the transcription/processing/transport machinery to form loose assemblies of complexes so large that they essentially become insoluble matrix structures. Consistent with this concept, members of the SR family of splicing factors, including ASF/SF2, are known to interact in a phosphorylation-dependent manner in vivo (Yeakley et al., 1999, and references therein). Many, though not necessarily all, of the components in this lattice-like matrix could be in flux, but the matrix itself might be relatively immobile. Such a model helps to explain an apparent paradox concerning the distribution of splicing factors. Both papers show that the ASF/SF2 concentrated in splicing factor domains (the more prominent speckles) moves at a rate similar to the more dispersed molecules in the rest of the nucleoplasm. But as is evident in Kruhlak's Figure 1 and in previous work (Misteli et al., 1997), most domains in a cell are relatively immobile. Thus, while factors within nuclear compartments may be in flux, the compartment itself is not. This contributes to the Kruhlak et al. (2000) interpretation that ASF/SF2 associates with structure.

It has been reported that clusters of factors can move vectorially from a larger domain, where it has been suggested they are stored, to a gene which was just induced (Misteli et al., 1997). Such observations might suggest an active and directed transport of splicing factors, possibly involving movement along a structure. However, results in these two papers report that splicing factor movement in general is neither energy dependent nor affected by phosphorylation, even though the latter has been shown to affect their overall distribution (Misteli and Spector, 1996; Yeakley et al., 1999). Kruhlak et al. (2000) attempt to study the movement of these tiny subdomains, but conclude that movement of such clusters is limited and not likely to be the major way in which factors find their targets. Since factors are sufficiently mobile to reach their targets by a stochastic and non-directed process, there may be no need for them to move along any kind of structure. But as Kruhlak et al. (2000) suggest, rather than diffusing freely, the movement of splicing factors may be constrained by frequent transient interactions with structure, which could still be important to their assembly and/or function.

Phair and Misteli (2000) discuss that diffusion of splicing factors may fit with other evidence for (pre)-mRNA within the nucleus. However, studies of (pre)-mRNA distribution illustrate the point that random dispersal and structural constraint are not mutually exclusive alternatives. Again, there is room for common ground. RNA distributions can vary with the type of RNA and even reflect different states of the same mRNA within a cell. There is evidence that some mRNAs randomly disperse (e.g., Singh et al., 1999), whereas others appear structurally constrained (Fig. 2, B and C; Lawrence et al., 1989; Lampel et al., 1997), and still others seem structurally constrained at some steps and more dispersed at others (Fig. 2 A; Xing et al., 1995). Using fluorescence correlation spectroscopy, Politz et al. (1998) found evidence that most nuclear poly A RNA moves at a rate close to diffusion, but that a significant component moves much more slowly, suggesting it may be tethered to nuclear structure. These different observations are not contradictory, but rather reflect complex biological realities. Since FRAP analysis provides only an average for the population (discussed in Politz, 1998), it remains to be determined if there are also different subpopulations of ASF.

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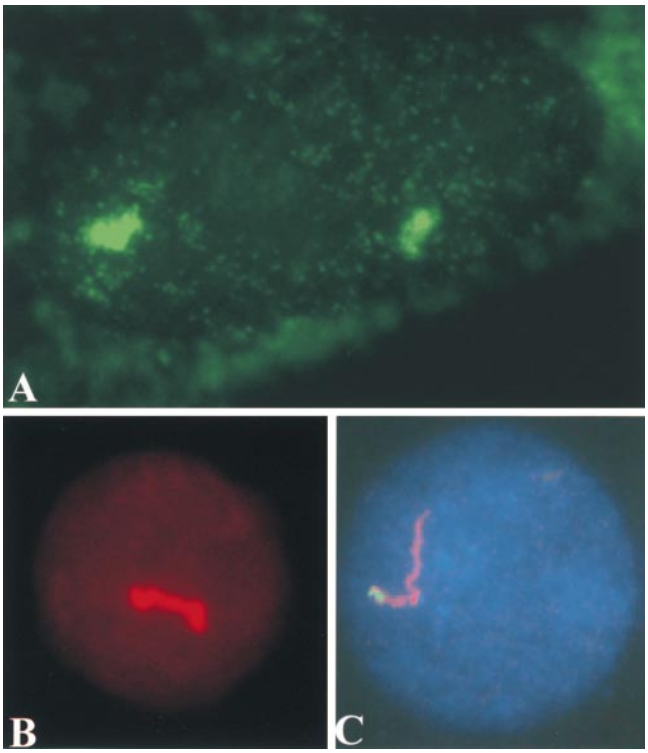

Figure 2. Reflecting real differences in the complex biology, distributions of different nuclear RNAs vary from dispersed to highly localized, or a combination of the two. (A) COL1A1 RNA shows two very localized accumulations in a diploid fibroblast nucleus as well as low level dispersed signal, likely representing individual transcripts. Other work has show that the localized accumulations are posttranscriptional transcripts emanating to one side of the gene, hence we refer to them as tracks (Smith et al., 1999, and references therein; Johnson et al., 2000). (B and C) In human lymphoma cells, RNA expressed from integrated Epstein Barr Virus produces a highly elongated curvilinear track, with no detectable evidence of free diffusion in the rest of the nucleoplasm. Tracks are apparent in many paraformaldehyde fixed cells (B), but become more elongated in nuclei swollen with hypotonic solution (C). Two-color analysis shows the gene (green) is consistently at one end of the much longer RNA track (red), as previously suggested (Lawrence et al., 1989) and recently shown (Melcak et al., 2000).

Do Splicing Factor Domains Behave as Stable Nuclear Structures?

If splicing factors exist in complexes of complexes of many different factors, at what point can one reasonably conclude that they are associated with a structure rather than macromolecular complexes diffusing in interchromosomal space? As many have suggested before us, a reasonable point of reference is when non-chromatin factors are no longer soluble or readily released from the nucleus. Because of the legitimate concern that salt extraction of unfixed cells could precipitate artifacts, a number of experiments reported years ago were designed to address those concerns for studies of the nuclear matrix in general (for example, Nickerson et al., 1997; Jackson and Cook, 1985). Since splicing factor rich domains and nuclear RNAs are easily visualized by fluorescence microscopy, their association with structure has been shown by a straightforward observation that gets too little attention: many RNA metabolic factors, as well as nuclear RNAs, are highly resistant to extensive detergent extraction (for example, see Carter et al., 1993, and references therein; Misteli and Spector, 1996). In contrast, cytoplasmic mRNAs (and many proteins) are much more fragile. Future FRAP studies might do well to include some analysis of simple solubility by this approach. We have found that even 18 h after detergent extraction, splicing factor rich domains in unfixed cells are still very much intact, even when the nucleus itself can become quite disrupted. Recent preliminary observations show that most factors do not just diffuse away when unfixed mammalian nuclei are mechanically disrupted (Shopland, L.S., and J.B. Lawrence, unpublished observations). Thus, the important finding of ASF/SF2's mobility in these domains does not negate the view that these comprise structures, consistent with earlier observations that these regions correspond to structures termed interchromatin granule clusters seen by electron microscopy (Fakan and Puvion, 1980).

A telling comparison is HM6-17 or fibrillarin, both of which show high mobility but are clearly known to be associated with structures.

How Do Splicing Factor Distributions Relate to Gene Expression?

The findings of Kruhlak et al., (2000) and Phair and Misteli (2000) leave open the question of why these factors form 20–40 distinctly higher-concentration domains, and how this might relate to their function. They find the vast majority of factors recover at similar average rates in both the concentrated domains and in the more dispersed nucleoplasmic pool. In our view, this observation is not easily reconciled with the idea that splicing factor rich domains contain a higher proportion of inactive factors, which are then directly recruited to distant sites of function randomly dispersed through the nucleoplasm. It is our perspective that an understanding of why and where these domains form cannot be answered without a detailed understanding of how nuclear metabolism of specific genes and RNAs is spatially arranged. Domains rich in splicing factors and poly A RNA are not randomly arranged but show a three-dimensional topography that is consistent for a given cell-type and related to the organization of the genome (Carter et al., 1993, and references therein). The high turnover of factors in domains shown by these authors fits the idea that factors within domains are in dynamic use, perhaps in rapid reassembly of complexes closely associated with expression of one or more highly active, complex genes (Smith et al., 1999; Johnson et al., 2000). The lower nucleoplasmic levels of factors may be sufficient for genes whose transcription does not outpace splicing (Smith et al., 1999), whereas concentrating factors in domains may serve to maximize expression of more demanding genes. This should not be construed to suggest that these distributions simply reflect the accumulation of dispersed factors on nascent pre-mRNAs of highly active genes. More consistent with earlier ultrastructural observations (Fakan and Puvion, 1980), detailed

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analysis of components within an individual domain suggest that they are far more complex structures (Johnson et al., manuscript submitted for publication).

A very recent review in *Science* discusses some of this work. It may be construed to convey that most key issues are resolved and that it is now well established that factors freely diffuse in nucleoplasmic space, and concentrate at sites of function (Lewis and Tollervey, 2000). However, we hope our discussion of the studies by Kruhlak et al. (2000) and Phair and Misteli (2000) makes clear that many compelling questions remain and that explanations should not be expected to be simple. Rather, common ground may only be found in the complexity of nuclear structure.

We thank John McNeil for assistance in preparing the figures. Comments have a restricted number of citations, hence we are unable to cite all relevant references. Our apologies to those authors whose work we have not specifically listed.

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